A Method for Producing Cross-linked Hyaluronic acid – Protein Bio-composites

BACKGROUND OF THE INVENTION

1.**Field of Invention**:

This invention relates generally to a new method for producing cross-linked hyaluronic acid – protein bio-composites in various shapes, and in particular, to a method for producing cross-linked hyaluronic acid – protein bio-composites from a homogenous solution preparing by mixing hyaluronic acid and protein at various ratios. The prepared bio-composites can be processed into different shapes.

2. **Description of the Related Art**

Hyaluronic acid (HA) is a muco- polysaccharide occurring naturally in and purified from the vertebrate tissues and fluid, and having a linear structure with high molecular weight from several thousands to several millions daltons depending on its source and purification method. Karl Meyer et al. in 1934 first reported that HA contains glucuronic acid and glucosamine and was isolated and purified from the vitreous humor of cow. HA is a linear chain polymer having repeat units of N-acetyl-D-glucosamine and D-glucuronic acid residues bonded through beta (1→3)bonding and then beta (1→4) bonding. HA is widely distributed in connective tissues, mucous tissue, crystalline lens and capsules of some bacteria. In commercial applications, HA has been used as a matrix in drug delivery, an arthritic agent, a healing agent for arthritic operation or general wound healing. In industrial production, HA was mainly extracted and purified from the cockscomb, but HA can also be isolated and produced from the capsules of *Streptococci spp.* by fermentation bio-technique.

HA aqueous solution shows both a high viscosity and flexibility. HA is generally called viscoelastic matrix when applied in the ophthalmology. The viscoelastic characteristic is attributed to the sponge polymeric network formed from bulk molecular volume HA having high MW. HA is

in vivo synthesized from HA synthetase that exists in the plasma membrane, and hydrolyzed by the hyaluronidase that exists in lysozyme. The interaction of HA and proteoglycans can stabilize the structure of resultant matrix and modify the behavior of cell surface. This characteristic exhibits many important physiological functions, including: lubrication, water-sorption, water retention, filtration, and modulates the distribution of cytoplasmic protein.

It is known that HA has functions of (1) naturally occurring in human body, (2) no immune reaction, (3) degradability and absorbability in human body, (4) easy availability, (5) a high molecular weight bio material applied in medicine. The major application of HA is in the ophthalmic operation of cataract and cornea damage. High molecular of aqueous HA solution is injected into eye as a viscoelastic fluid to maintain the morphology and function of eye. HA has been recently applied in wound healing, tissue anti-adhesion after surgery and drug delivery applications. HA is present in intra-cells as a complex with protein in tissue, which forms a jelly matrix owing to it high water retention and can thus be useful in cosmetic application as an anti-aging agent.

Collagen is a structure protein found in animals. It is a naturally occurred biopolymer, and its moiety causing an immune-reaction could be eliminated via isolation, purification and optional treatment with enzyme (such as pepsin), to give collagen having a good bio-compatibility. Collagen can be processed by various reconstruction, chemical cross-linking reaction and optional additional processing procedure to form into different shapes, such as plate, tube, sponge, powder or soft fabric. Since collagen will be biodegraded in vivo and is a low toxic polymer having excellent bio-compatibility in human body, it has been used as a hemostatic agent, nerve regenerating agent, tissue anaplastic agent, scald dressing material, hernia repair, urethra operation, drug delivery, ophthalmology, vaginal contraceptive, cardiac valve repair, blood vessel operation and operating structure, and other biomedical materials.

Gelatin is a denatured collagen. Its amino acid content is similar to the collagen but different in structure and chemic-physical properties. Up to date, it has been used in a wide variety of food application and medical research, such as hemostatic cotton and drug delivery.

HA and collagen are the major components of extra-cellular matrix. Gelatin is also made from collagen. Therefore, gelatin also has good bio-compatibility and biodegradation in human body. The gelatin composites can also be used for the development of implant matrices in biomedical materials field, such as histological engineering, active ingredient releasing system or as materials for preventing tissue from sticking after surgery.

(1) Milena Rehakova et al., 1996, Journal of biomedical materials research, vol. 30, pages 369-372,describes a method for preparing collagen and hyaluronic acid composite materials through the use of glyoxal and starch dialdehyde as a cross-linking agent. The collagen was dispersed in 0.5M acetic acid solution, and then HA was added to the solution and reacted for 5mins. Fiber was precipitated and filtered, washed several times with water and alcohol, and dried at a temperature of 35℃, and then a fiber structure in the form of a film having a smooth surface was produced. The cross-linking of the composite material was carried out in the presence of an aqueous starch dialdehyde solution. In the case of using glyoxal as the cross-linking agent, the cross-linking was carried out by adding HA and glyoxal to the suspension of collagen, or adding glyoxal to the suspension of collagen first and then adding HA.

(2) Jin-Wen Kuo et al., 1991, Bio-conjugate chemistry, vol,2, pages 232-241, describes a method for preparing water-insoluble derivatives of hyaluronic acid by reacting high molecular HA with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at a pH of 4.75. In a general experiment, sodium hyaluronate was dissolved in distilled water to produce a 4 mg/ml HA solution. In some reaction, amine and sodium hyaluronate were added into the HA solution and mixed together. The pH of the aqueous solution was adjusted to pH 4.75. Carbodiimide was dissolved in either water or isopropanol, depending on the solubility of carbodiimide.

After mixing of HA and carbodiimide, the resultant solution was maintained at a pH of 4.75 by addition of 0.1N HCl using a pH meter. The reaction mixture was kept at room temperature for 2 hrs, then HCl solution was added until a concentration of HCl was 5% (w/v) in the solution, and then a precipitate is formed after adding 3 time volume solution of ethanol. Non-reacted chemical reagent was washed out for 2-3 times with distilled

water. Finally, the precipitate was dissolved in deionozed water before lyophilization.

(3) Lin-Shu Liu et al., 1999, Biomaterials, vol, 20, pages 1097-1108, states a method for preparation of hyaluronate-polyaldehyde by treatment of hyaluronate with sodium periodate. Hyaluronate-polyaldehyde was prepared by oxidizing sodium hyaluronate with sodium periodate. A collagen-hyaluronate matrix was synthesized by covalent bonding of aldehyde group to the collagen to obtain a material for supporting cartilage tissue or repairing bone.

(4) D. Bakos et al., 1999, Biomaterials, vol, 20, pages 191-195, describes a new method for preparing bio-composite material. The composite material consisted of nine parts by weight of inorganic component hydroxyapatite and one part of organic component including 92wt% collagen and 8wt% hyaluronic acid. Hydroxapatite particles was gradually added to the solution of hyaluronic acid in de-ionized water, and intensively stirred and mixed. Separately, very fine collagen fibers (1% by dry weight) was dispersed in de-ionized water after dry fibrillation of lyophilized fibers of collagen. The two prepared dispersions were mixed together to form complex precipitate. The precipitate was filtered and dried at a temperature of 37℃to obtain a composite which did not undergo any cross-linking reaction.

(5) C. J. Doillon et al., 1988, Biomaterials, uses a porous sponge of collagen as a support for the growth of epithelium and fibroblast cell, and as a matrix of artificial skin. HA and/or fibronectin can enhance the repair on wounded skin and the proliferation of cell. These high molecular can modify the behavior of cell in tissue culture. The method of preparation includes a step of dispersing water-insoluble collagen (1% by weight) in hydrochloric acid solution at a pH 3.0. In this step, 1%w/w of hyaluronic acid, fibronectin, dermatan sulfate and chondroitin-6-sulfate were added to the collagen solution. The dispersion solution was frozen at -30℃,and then lyophilized before cross-linking.

(6) S. Srivastava et al., 1990, Biomaterials, vol, 11, pages 155-161, indicates that collagen gels modified or added with glucosaminoglycans, (e.g. 5% or 10% chondroitin sulfate or less than 5% of HA) would enhance the cell growth and adhesion, the growth and adhesion of cells

would be inhibited if more than 5% HA was incorporated into collagen gels.

(7) S. Srivastava et al., 1990, Biomaterials, vol, 11, pages 162-168, studied the effect of the collagen or modified collagen on the growth of fibroblast cell line. The preparation of collagen/GAGs and fibronectin composite materials were following the method described by Yannas. 3%w/v of degassed collagen slurry was stirred in 0.05M acetic acid while a solution of HA dissolved in 0.05M acetic acid was added to the resultant solution until the dry weight of GAGs was 2.5% based on the weight of collagen, and then solution was homogenized and degassed. Collagen/HA composite material contains 5%, 10%, or 20% GAGs, and collagen/CS composite material contains 5% or 10% chondroitin-4-sulfate and chondroitin-6-sulfate. Their preparation method was the same as the above described. 1% Fibronectin was further added to the above composite material, and placed on the petri dish for culturing cell. Experimental results showed that polystyrene was better than nature collagen to be a material of petri dish, but the adhesion of collagen was improved by chemical modification or by adding with fibronectin and chondroitin-4-sulfate. If content of HA was more than 5%, however, the cell adhesion and growth of nature collagen matrix could be better than the polystyrene material.

(8) M. Hanthamrongwit et al., 1996, Biomaterials, vol, 17, pages 775-780, studies the effect of the glycosaminoglycans, hyaluronic acid and chondroitin-6-sulfate, diamines and carbodiimides cross-linking agents on the growth of human epidermal cells in collagen gels. Collagen gel (0.3% w/v) was prepared by mixing 4.2 mg/ml collagen solution, a mixture of 10times of volume of DMEM and 0.4M NaOH (2:1), and 1:100 (v/v) acetic acid at a ratio of 7:1:2, and adjusting the solution at pH 8-8.5 by addition of 1M NaOH. The gels were stood for 2hrs at room temperature. If intend to add GAG, hyaluronic acid and chondroitin-6-sulfate solutions in serum-free DMEM substituted for acetic acid used in the above solution at various ratio. After forming gels, 1-ethyl-3- (3-dim hyaluronic acid and chondroitin-6-sulfate ethylaminopropyl carbodiimide) and diamine were incorporated into the gels to subject to cross-linking reaction.

(9) L.H.H. Olde Damink et al., 1996, Biomaterials, vol, 17, pages 765-773, describes that non-cross-linked dermal sheep collagen, (N-DSC) was cross-linked with EDC to give E-DSC by immersing 1g N-DSC

samples (1.2mmol) in 100ml of an aqueous solution containing 1.15g (6.0mmol) EDC at room temperature for 18hrs. During the reaction, a pH of the solution was maintained at 5.5 by addition of 0.1M HCl using a pH meter. The molar of N-DSC samples was calculated assuming that 120 carboxylic acid group residues are present per α-chain (~1000 amino acids) and that each α-chain has a molecular weight of 100,000. After cross-linking, E-DSC samples were washed for 2hrs in a 0.1M Na_2HPO_4 solution and subsequently washed four times with distilled water before lyophilization. The other cross-linking of N-DSC with EDC and NHS to give E/N-DSC was performed by immersing N-DSC samples in aqueous solution containing EDC and NHS at room temperature for 4hrs. The results showed that addition of N-hydroxylsuccinimide to the EDC-containing cross-linking solution (E/N-DSC) increased the rate of cross-linking.

(10) Yannas et al., 1997, U.S. Pat. No. 4,060,081, states a multilayer membrane which is useful as synthetic skin. Preferred materials for the first layer are cross-linked composites of collagen and a muco-polysaccharide. A second layer is formed from a nontoxic material which controls the moisture flux of the overall membrane.

(11) Yannas et al., 1981, U.S. Pat. No. 4,280,954, states a method for preparing cross-linked collagen-muco-polysaccharide composite materials. A collagen solution at pH 3.2 and muco-polysaccharide solution (weight ratio is 6%-15% by weight) were mixed together, and then a precipitate of aldehyde covalent cross-linked collagen-muco-polysaccharide composite was formed.

(12) Yannas et al., 1982, U.S. Pat. No. 4,350,629 discovers that if collagen fibrils in an aqueous acidic solution (<pH 6.0) are contacted with a cross-linking agent (glutaraldehyde) before being contacted with glycosaminoglycan, the materials produced have extremely low level of thrombogenicity. Such materials are well suited for in-dwelling catheters, blood vessel grafts, and other devices that are in continuous contact with blood for long periods of time.

(13) Yannas et al., 1984, U.S. Pat. No. 4,448,718, describes a process for preparing a cross-linked collagen- glycosaminoglycan composite material which comprises forming an uncross-linked composite material from collagen and a glycosaminoglycan and containing the uncross-linked

composite with a gaseous aldehyde until a cross-linked product having an M. sub. C of from about 800 to about 60,000 is formed.

(14) Balazs et al., 1986, U.S. Pat. No. 4,582,865, states a method for preparing cross-linked gels of hyaluronic acid and products containing such gels. The cross-linking HA or HA/hydrophilic polymers (polysaccharide or protein)and the divinyl sulfone was carried out at 20℃ in a pH＞9 solution. In the 1%-8% dry solids content of mixture, HA contains 5%-95% of dry solids content.

(15) Liu et al., 1999, U.S. Pat. No. 5,866,165, states a matrix and a method for preparing it, which matrix is provided to support the growth of bone or cartilage tissue. A polysaccharide is reacted with an oxidizing agent to open sugar rings on the polysaccharide to form aldehyde groups. The aldehyde groups are reacted to form covalent linkages to collagen. Collagen and polysaccharide used to form matrix are present in a range of 99:1 to 1:99 by weight, respectively. 1% to 50% of the repeat units in polysaccharide are oxidized to contain aldehyde groups.

(16) Pitaru et al., 1999, U.S. Pat. No. 5,955,438, states a method for producing a collagen matrix which may be formed into a membrane useful in guided tissue regeneration. A collagen matrix comprises collagen fibrils are incubated with pepsin in a solvent, and are then cross-linked to one another by a reducing sugar. Finally, the matrix is subjected to critical point drying.

(17) Pierschbacher et al., 1999, U.S. Pat. No. 5,955,578, states a method for producing polypeptide-polymer conjugates active in wound healing. A synthetic polypeptide comprising the amino acid sequence dArg-Gly-Asp is bonded to a biodegradable polymer via a glutaraldehyde cross-linking agent. The purpose of synthetic matrix is to promote cell attachment and migration.

(18) Hall et al., 1998, U.S. Pat. No. 5,800,811, states a method for producing an artificial skin. An artificial skin is prepared by impregnating a collagen with a transforming growth factor-beta, and incubating the impregnated matrix with a source of stem cells.

(19) Stone et al., 1989, U.S. Pat. No. 5,880,429, states a method for producing a prosthetic meniscus. A pore size in the range 10-50 microns

of prosthetic meniscus is formed by type I collagen fibrils (65%-98% by dry weight) and glycosaminoglycan molecular (chondroitin-4-sulfate ; chondroitin-6-sulfate ; dermatan sulfate or hyaluronic acid ; 1%-25% by dry weight) and which is adapted for in growth of meniscal fibrochondrocytes.

(20) Stone, 1992, U.S. Pat. No. 5,108,438, states a method for producing a prosthetic inter-vertebral disc. The disc includes a dry, porous, volume matrix of bio-compatible and bio-degradable fibers which may be interspersed with glycosaminoglycan molecules (0-25% by dry weight) .

The cross-linking agent is selected from the group consisting of glutaraldehyde, carbodiimides and so on.

(21) Silver et al., 1987, U.S. Pat. No. 4, 703,108, states a method for preparing biodegradable collagen-based matrix in sponge or sheet form. HA and collagen are added to a dilute HCl solution of pH 3.0 and the mixture is homogenized in a blender. The solution is then poured into a vacuum flask and de-aerated at a vacuum, and then cross-linked with carbodiimide. After then, the matrix is allowed to air dry or freeze dry. The product of collagen-based matrix is cross-linked by immersion in an aqueous solution containing 1% by weight of cyanamide at pH 5.5 for a period of 24hrs at 22℃. Hereinafter, the matrix is frozen and dried at -65 ℃ in a vacuum.

(22) Silver et al., 1990, U.S. Pat. No. 4,970,298, states a porous biodegradable collagen sponge-like matrix which enhances the healing of wound. Collagen is dispersed in an acid solution of pH from 3.0 to 4.0 and mixed with the fibronectin in an acid solution of pH 3.0 to 4.0 in a blender. Collagen dispersions to be converted into sponge are frozen at -100℃ before freeze drying at -65℃. The matrix is cross-linked in two steps consisting of first cross-linking with carbodiimide and then subjecting to dehydrothermal, or first subjecting to dehydrothermal and then cross-linking with carbodiimide.

SUMMARY OF THE INVENTION

Based on the reports of patents and references above-mentioned, the general preparation of the polysaccharide-protein bio-composites is under

an acid condition, a polysaccharide-protein fiber precipitate is formed by forming ionic bond between polysaccharide and protein from mixing minor amount of polysaccharide (less than 15% weight of collagen) and protein, and the resultant precipitate is further cross-linked with a cross-linking reagent to form the covalent bond, a non-directional fiber sponge or porous matrix is produced after washing, filtration and lyophilization. A defect of this process is only that a non-homogeneous porous matrix having fiber structure, other than a homogenous composite, can be produced, it is difficult to form impalpable matrices in a suitable shape as desired. If a shape is required, a piece of the prepared precipitate is generally homogenized by chopping it into small segments, and the homogenized slurry was then poured into a mold having the desired shape, then lyophilized. According to method of the present invention, a mixture solution consisting of polysaccharides and protein at various ratio having different pH value is prepared, and then processed into bio-composites having different shapes as desired (such as membrane, sponge, fiber, tube or micro-granular and so on). Subsequently, the bio-composites is subjected to a cross-linking reaction in a solution of water and organic solvent to obtain impalpable bio-composites which is a homogeneous, bio-compatible, biodegradable and has excellent physical properties and a prolonged enzymatic degradation time.

The advantage of this invention is that a homogenous polysaccharide-protein solution can be prepared under a wide range of pH value, not only under an acid condition, and the weight ratio of polysaccharide to protein is from 2/98 to 90/10. In traditional methods, the collagen is usually used as a major component and the polysaccharide is used as an additive, the maximal ratio of polysaccharide to collagen is around 20%. Besides, the matrix solution produced from this invention possesses a uniform density and a porosity, and can be manufactured into various shapes, including membrane, sponge, fiber, tube or fine particles and so on. It can also avoid the loss of polysaccharide and reduce reaction time to only 2-4hrs if a cross-linking reaction with carbodiimide is conducted in the presence of weak acid in organic solvents.

In prior arts, it usually uses aldehydes as a cross-linking reagent. If carbodiimide is used as a cross-linking agent, the cross-linking reaction is

always conducted in water and will take place for more than 24hrs.

There are many advantages in this invention. The techniques of the present invention have never been described in previous references. According to the method of the present invention, bio-composites which can be produced in various shape are suitable for using in a variety of fields, including biomedical, materials engineering, histological engineering, medical equipment, pharmacy and cosmetic fields.

Other features and advantages of the present invention will be apparent from the following description of the preferred embodiments thereof and the appended claims.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to a method for producing polysaccharide-protein bio-composites having various shapes. The advantage of the method is that the bio-composite can be produced into various shapes, such as membrane, sponge, fiber, tube, and micro-granular. After further subjecting to cross-linking reaction, a bio-composite which is bio-compatible, biodegradable, non-toxic, and impalpable, and possesses prolonged enzymatic degradation time and excellent mechanical strength is formed. It is extremely suitable for the application in biomedicine, histological engineering, materials engineering, medical equipment and cosmetic fields. The bio-composite prepared by the present method is suitably used as hemosats, vascular sealants, orthopedic implant coatings, vascular implant coatings, dental implants, wound dressings, anti-adhesion barriers, platelet analyzer reagents, research reagents, engineering of cartilage, artificial tendons, blood vessels, nerve regeneration, cornea implants, cell preserving solutions and for delivering growth factor and/or drugs. According to the present invention, the prepared bio-composite can be further processed into various products possessing high additional value. It is very useful for commercial utilization.

The present invention relates to a method for producing polysaccharide-protein bio-composites, comprising the steps of:

(a) preparing a polysaccharide aqueous solution;

(b) preparing a protein aqueous solution;

(c) well-mixing the solutions from steps (a) and (b), adjusting a pH of the mixture in a moderate range by adding either an acid or a hydroxide, processing the mixture into matrix having a desired shape, such as membrane, porosity, sponge, tube or micro-granular and so on;

(d) subjecting the matrix to cross-linking reaction in a mixture of water and organic solvents containing across-linking reagent under a pH of from 4 to 4.5 at a temperature of from 20 to 45℃ for a period of 1 to 6 hours, preferably 2 to 4 hours;

(e) washing the matrix for several times, and immersing in a salt aqueous solution which is chosen from the group consisting of sodium chloride, dibasic sodium phosphate or a mixture thereof.

The matrix was then further washed several times with large volumes of de-ionized water and lyophilized.

In the Step(a), the polysaccharide is chosen from the group consisting of hyaluronic acid, carboxymethyl cellulose, pectin, starch, chondroitin-4-sulfate, chondroitin-6-sulfate, alginate, chitosan, agar, carragenan and guar gum, and a mixture thereof.

In the step (b), the protein is chosen from the group consisting of collagen, gelatin, or a mixture thereof.

In the step (c), the preferred pH value is in a range between 3 and 11,and if an intended pH is less than 7, it is adjusted by adding acetic acid, hydrochloric acid, or a mixture thereof. If an intended pH is more than 7, it is adjusted by adding sodium hydroxide, potassium hydroxide, or a mixture thereof.

The solids content of resultant polysaccharide-protein mixture is in a range between 0.2% and 4.0% by weight, and the percent of polysaccharide is in a range between 2% and 98%, based on the total weight of the mixture.

As to the procedures for forming the matrix into different shapes in the step (c) are illustrated in details as follows:

(1) The matrix is prepared as a film matrix by casting the degassed matrix consisting of polysaccharide and protein solutions into a mold and drying in an oven at a temperature of 35℃ to yield a film matrix.

(2) The matrix is prepared as a porous matrix by casting the degassed matrix consisting of polysaccharide and protein solutions into a mold in a refrigerator at a temperature of -80℃and drying at a vacuum to yield a porous matrix having a inter-connective porous structure.

(3) The matrix is prepared as a powder matrix by dropping the degassed matrix consisting of polysaccharide and protein solutions into the freezing solution at a temperature of -80℃ by using a syringe, and lyophilizing under a vacuum to yield powder matrix.

(4)The matrix is prepared as a fiber matrix by squeezing the degassed matrix consisting of polysaccharide and protein solutions into a coagulant solution in a mixture of water and organic solvents, and lyophilizing to yield a fibrous matrix having a thickness of from 50um to 1mm.

The organic solvent contained in the coagulant solution is chosen from the group consisting of 1,4-dioxane, chloroform, methylene chloride, N,N-dimethylformamide, N,N-dimethylacetamide, ethyl acetate, acetone, methyl ethyl ketone, methanol, ethanol, propanol, isopropanol, butanol, and a mixture thereof ; the percentage of the organic solvent in the coagulant solution is between 60% and 100% by weight, preferably between 75% and 100% by weight. The preferred organic solvent is a mixture of ketones and alcohols.

The cross-linking agent in step (d) is preferably a carbodiimide, which is selected from the group consisting of 1-methyl-3-(3-dimethylaminopropyl)- carbodiimide, 3-(3-dimethylaminopropyl)-3-ethyl-carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide or any mixture thereof.

The mixture of water and organic solution in the step (d) is preferably consisting of 5%-50% by weight of water and 95-50% by weight of either ethanol or acetone, or the both, preferably consisting of 5%-30% by weight of water and 95-70% by weight of either ethanol or acetone, or the both.

The salt aqueous solution in step (e) is used at a concentration of

0.15-4M. The immersion time is in a range between 30mins and 3hrs.

The present invention is described in more detail in the following example. These examples are giving by way of illustration and are not intended to limit the invention except as set forth in the claims.

Example 1A-1G: Preparation of hyaluronic acid/collagen matrix

Hyaluronic acid (HA)(60mg)and collagen(40mg)were each dissolved indifferent solvent as shown in table 1 described) , and then the prepared two solutions were mixed together to form a mixture that a weight ratio of HA to collagen is 3 to 2 and a solid content of the mixture is 1%.

The resulting mixture was cast into a mold made of Teflon to yield a film. The films prepared in Example 1D and 1E had the optimal morphology and physic properties.

Table 1

Example	1A	1B	1C	1D	1E	1F	1G
HA[a] solvent	H_2O	0.1N NaCl	0.1M CH_3COOH	H_2O	H_2O	H_2O	H_2O
Collagen	0.5M CH_3COOH	0.1M CH_3COOH	0.1N NaCl	0.1M CH_3COOH	Dissolving in 0.5M acetic acid, Then adjusting pH by 1N NaOH	Dissolving in water and then adjusting pH 7 by HCl	A mixture of 0.5M CH_3COOH and 1N NaOH
NaCl	-	-	-	30mg	-	-	-
mixed solution	white fiber precipitate	transparence and low viscosity	transparence	transparence	transparence	fine fiber precipitate	white fiber precipitate
1N NaCl	few drops, fiber precipitate and then dissolved	-	-	-	-	-	-
PH	~9	~8	~7	~3	~6	~7	~6
morphology	fine fiber on the matrix surface	semi transparence	semi transparence	white and dense	White, dense and high toughness	fine fiber on the matrix surface	white

Example 2: Preparation of HA/gelatin matrix

HA (50mg) was dissolved in 5ml of pure water. Separately, gelatin (50mg) was dissolved in 5ml of warm water (more than 55℃) and then added with sodium chloride (30mg).

The prepared two solutions were mixed together to form a 10ml mixture of which pH is around 6.5, the weight ratio of HA to collagen is 1 to 1 and a solid content is 1%.

The resulting solution was cast into a mold made of Teflon and allowed to dry in an oven to yield a transparent film.

Example 3: Preparation of HA/collagen matrix at different salt concentration after neutralization.

HA(60mg)was dissolved in pure water. Separately, collagen(40mg) was dissolved in 0.5M acetic acid solution, and then neutralized with sodium hydroxide. Adjust the salt concentration after neutralization and maintain the pH at 6 by adding various volume of water, acetic acid and sodium hydroxide as shown in Table 2. The prepared two solutions were mixed together to form a 10ml mixture in which a weight ratio of HA to collagen is 3 to 2 and a solid content is 1%.

The resulting solution was cast into a mold made of Teflon and allowed to dry in an oven to yield a film.

Table 2

Example	3A	3B	3C
H_2O (ml)	5.5	7.0	8.5
0.5M CH_3COOH	3.0	2.0	1.0
1N NaCl	1.5	1.0	0.5
Salt conc of neutralization. (M)	0.15	0.1	0.05

Example 4: Preparation of HA/collagen matrix at different pH

HA(60mg)was dissolved in pure water. Separately, collagen(40mg) was dissolved in 0.5M acetic acid solution, and then neutralized with sodium hydroxide. Adjust a pH value by adding various volume of acetic acid and sodium hydroxide as shown in Table 3 and maintain a salt concentration after neutralization at 0.15M. The prepared two solutions were mixed together to form a 10ml mixture in which a weight ratio of HA to collagen is 3 to 2 and a solid content is 1%.

The resulting solution was cast into a mold made of Teflon and allowed to dry in an oven to yield a transparent film.

Table 3

Example	4A	4B	4C
H_2O (ml)	3.5	5.5	5.44
0.5M CH_3COOH	5.0	3.0	3.0
1N NaCl (ml)	1.5	1.5	1.56
PH value	4.7	6.0	11.0

Example 5: Preparation of HA/collagen matrix at different ratio

HA was dissolved in pure water. Separately, collagen was dissolved in 0.5M acetic acid solution, and then neutralized with sodium hydroxide. A salt concentration after neutralization is maintained at 0.15M, a pH is maintained at 4.7, and the volume ratio of added water, acetic acid and sodium hydroxide is maintained at 3.5:5:1.5. The prepared two solutions were mixed together to form a 10ml mixture in which a weight ratio of HA to collagen is as shown in Table 4 and a solid content is 1%.

The resulting solution was cast into a mold made of Teflon and allowed to dry in an oven to yield a transparent film.

Table 4

Example	5A	5B	5C	5D	5E	5F
HA (mg)	90	80	60	50	20	2
Collagen (mg)	10	20	40	50	80	98
Weight ratio (HA/collagen)	9:1	4:1	3:2	1:1	1:4	1:49

Example 6: Preparation of HA/collagen matrix at different solid content

HA was dissolved in pure water. Separately, collagen was dissolved in 0.5M acetic acid solution, and then neutralized with sodium hydroxide. Maintain a salt concentration after neutralization at 0.15M and a pH at 4.7, the volume ratio of added water, acetic acid and sodium hydroxide is at 3.5:5:1.5. The prepared two solutions were mixed together to form a 10ml mixture in which a weight ratio of HA to collagen is 3 to 2 and a solid content is as shown in Table 5.

The resulting solution was cast into a mold made of Teflon and allowed to dry under oven to yield a transparent film.

Table 5

Example	6A	6B	6C
HA (mg)	120	60	30
Collagen (mg)	80	40	20
Solid content (%)	2	1	0.5

Example 7: Preparation of HA/collagen matrix in a fiber form

HA (100mg) was dissolved in 3.5ml of pure water. Separately, collagen (100mg) was dissolved in 5ml of 0.5M acetic acid solution, and then neutralized with 1.5ml of 1N sodium hydroxide. The salt concentration of neutralization is 0.15M. The prepared two solutions were mixed together to form a mixture in which a pH of solution is around 4.7, a weight ratio of HA to collagen is 1 to 1 and a solid content is 2%.

The resulting solution was continuously pressed into a 95% alcohol solution to form a mono-filament fiber by using syringes having various sizes, and allowed to dry in an oven to yield a HA-protein matrix.

Example 8: Preparation of HA/collagen matrix in a form of micro-granular

HA (100mg) was dissolved in 3.5ml of pure water. Separately, collagen (100mg) was dissolved in 5ml of 0.5M acetic acid solution, and then neutralized with 1.5ml of 1N sodium hydroxide. The salt concentration after neutralization is 0.15M. The prepared two solutions were mixed together to form a mixture in which a pH of the mixture is around 4.7, a weight ratio of HA to collagen is 1 to 1 and a solid content is 2%.

The micro-granular matrix was formed by dropping the resulting mixture into the liquid nitrogen and lyophilized.

Example 9: Preparation of HA/collagen matrix in a porous form

HA (100mg) was dissolved in 3.5ml of pure water. Separately, collagen (100mg) was dissolved in 5ml of 0.5M acetic acid solution, and then neutralized with 1.5ml of 1N sodium hydroxide. The salt concentration after neutralization is 0.15M. The prepared two solutions were mixed together to form a mixture in which a pH of solution is around 4.7, a weight ratio of HA to collagen is 1 to 1 and a solid content is 2%.

The resulting solution was cast into a mold made of Teflon at a temperature -80℃ and allowed to dry to yield a porous sponge matrix after lyophilization.

Example 10: The effect of cross-linked agent on cross-linking reaction of HA/collagen matrix.

The film of Example 6A was chopped to pieces in equal size and immersed in the EDC to subject to cross-linking reaction at 30℃ for 2 hours (experimental conditions were shown in Table 6). The mixture was then washed 3 times with 80% acetone aqueous solution, each washing time is 20mins. After then, the mixture was further washed 3 times with de-ionized water, each washing time is also 20mins. Finally, the mixture was spread on a substrate and dried. The cross-linked film was subject to swelling test by immersing in 0.15M sodium chloride solution, incubating for 5 days with gentle shaking at 37℃, then the swelling behavior was observed. From the results shown in Table 6, it showed that in order to avoid the dissolution of matrix and enhance the cross-linking efficiency, the cross-linking of matrix was only carried out in a mixture of water and organic solvent (Examples 10D,10E).

Table 6

Example	10A	10B	10C	10D	10E
EDC conc. (wt%)	2.3	2.3	2.3	2.3	2.3
Solvent	H_2O	PH4.7 solution	PH4.8 solution	80% ethanol	80% acetone
Morphology	thinness	thinness	thinness	normal	normal
Dissolving test	Soluble	soluble	soluble	insoluble	insoluble

Example 11: The effect of a concentration of cross-linked agent on the cross-linking reaction of HA/collagen matrix.

The film of Example 6A was chopped to pieces in equal size and immersed in 80% acetone solution containing EDC at pH 4.7 and at 30℃ for 2hrs (experimental conditions were shown in Table 7). The mixture was then washed 3 times with 80% acetone solution, each washing time is

20mins. After then, the mixture was further washed 3 times with de-ionized water, each washing time is also 20mins. Finally, the mixture was spread on a substrate and dried. The cross-linked film was subjected to swelling test by immersing in 0.15M sodium chloride solution, incubating for 5 days with gentle shaking at 37℃, then the swelling behavior was observed. Hyaluronidase (220U/ml) was dissolved in 0.15M sodium chloride. Film was weighted and put into the enzyme solution for testing enzyme degradability of the film. After 24 hours, the solution was taken out for uronic acid assay, and then the percent of hydrolysis of HA film was calculated. From the results in Table 7, it showed that the rate of enzyme degradation of the cross-linked film prepared by the present method was reduced significantly.

Table 7

Example	11A	11B	11C	11D	Control
EDC (wt%)	0.625	1.25	2.5	5	-
Dissolving test	insoluble	insolubl e	insolubl e	insoluble	soluble
HA enzyme degradation (%)	1.87	1.5	0.68	1.02	31.13

Example 12: Cross-linking reaction of a porous HA/collagen sponge matrix.

The porous sponge of Example 9 was placed in an oven at 110℃ and under a vacuum for 3hrs. The dried specimens were then immersed in a 80% acetone solution for 30mins, and then transferred to a 80% acetone solution containing 2.5% EDC at pH 4.7.

The specimens were taken out after reaction at 30℃ for 2 hours, and then washed 3 times with 80% acetone, each washing time is 20mins. After then, the specimens were further immersed in 1M sodium chloride for 20mins, and washed 3 times with deinoized water, each washing time is also 20mins. Finally, the specimens were spread on a substrate and dried.

Example 13: Determination of ability of growth of cell and cyto-toxicity of cross-linked HA/collagen.

The films prepared from Examples 5C, 5D and 5E were immersed in the 80% acetone solution containing 2.5% EDC at pH 4.7. The film was taken out after reaction at 30℃ for 2 hours, and then washed 3 times with 80% acetone, each washing time is 20mins. After then, the film was further immersed in 1M sodium chloride for 20mins, and washed 3 times with de-ionized water, each washing time is also 20mins. Finally, the film was spread on a substrate and dried.

The cross-linked film matrix was placed in a cell culture plate. Immortalized mouse 3T3 fibroblast cell and human fibroblast cell were seeded on the film matrix for observing the growth of cell (Please refer to Tables 8,9). The results of cell seeding experiment showed that cell can growth well on the film matrix, and all the cells were alive. Also, it is stained with neutral red dye and showed that the film matrix was non-toxic to the human and mouse cell growth.

Table 8

Example	Seeding of cell No ($x10^4$cell/ml).	1st day ($x10^4$cell/ml)	2nd day ($x10^4$cell/ml)	Third day ($x10^4$cell/ml)
Cross-linked 5C	4	1.8	2.4	4.8
Cross-linked 5D	4	2.4	4.2	7.4
Cross-linked 5E	4	1.4	1.8	3.4

Table 9

Example	Seeding of cell No ($x10^4$cell/ml).	1st day ($x10^4$cell/ml)	2nd day ($x10^4$cell/ml)	Third day ($x10^4$cell/ml)
Cross-linked 5C	4	1.2	2.2	5.0
Cross-linked 5D	4	2.6	4.4	7.4

Cross-linked 5E	4	1.6	2.4	4.0

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made by those skilled in the art without departing from the invention. Accordingly, the scope of the present is limited by the following claims.